Supplementary Materials for

Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors.

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Materials and Methods

Animals

Aged C57Bl/6 mice (15-16 and 21 months) were obtained from the National Institute on Aging (NIA). Young C57Bl/6J, B6.SJL and GFP mice (2 months) were obtained from Jackson Laboratories (USA). All animal studies were approved by Harvard University Institutional Animal Care and Use Committee and performed in accordance with institutional and federal guidelines.

Parabiosis, BrdU pulse and blood chimerism analysis

Parabiosis was performed as described previously (*33*) on young (2 months) and old (15-16 months) male mice. Experiments in which older (21months) mice were used are coded as Iso-O_{21} and Het-O_{21}. Het-Y_{21} indicates a Het-Y animal in parabiosis with an Iso-O_{21} mouse. To confirm parabiotic cross-circulation, we used young mice carrying the congenic marker CD45.1 and old mice carrying the congenic marker CD45.2 in the heterochronic pair and young mice carrying either CD45.1 or CD45.2 in the Iso-Y pair (Fig.S1). For blood chimerism analysis, spleens were dissected from the mice, the tissue homogenized and erythrocytes lysed. Cells were stained with an antibody cocktail (CD45.2-FITC, CD45.1-PE, Terr119-APC, PI). The frequency of chimerism was measured by flow cytometry and analyzed using FlowJo software (TreeStar, USA). Since old $CD45.1^+$ are not commercially available, we only used $CD45.2^+$ in the Iso-O pair, but cross-circulation in these mice has previously been described (*11*).

For reversal of parabiosis, mice were anesthetized with isoflurane. The skin was incised at the joining wound, separating the animals. The resulting incisions in the corresponding lateral aspects of separated mice were closed by staples or interrupting sutures (5-0 coated vicryl), and animals were given buprenorphine every 12 hours for at least 48 hours after surgery.

For the BrdU pulse 2-month old and 15-month old mice were used. One week after joining each animal in the parabiotic pair, called a parabiont, was injected with BrdU (50mg/kg) and pulsed every 12h for 3 days to label newborn neurons. Mice were sacrificed and analyzed after a total of 5 weeks since the parabiotic surgery.

Immunohistochemistry

Mice were perfused transcardially with 50ml PBS, followed by 50ml of 4% paraformaldehyde (PFA) and their brains were removed and post-fixed overnight in 4% PFA. Each brain was embedded in 4% agarose and 100µm-thick coronal sections were cut in a vibrating microtome (VT1000S, Leica) and pre-incubated in 10% normal goat or donkey serum, Triton 0.1% in PBS for 1h. For BrdU detection, sections were incubated for 40min with 2N HCl prior to blocking. Tissue sections or cell cultures were incubated overnight at 4°C with the following antibodies: rat monoclonal anti-BrdU (Abcam), mouse monoclonal anti-Olig2 (Millipore), rabbit polyclonal anti-Sox2 (Cell Signaling Technology), polyclonal rabbit anti-Ki67 (Abcam), rat monoclonal anti-CD31 (BD Biosciences), chicken polyclonal anti-GFAP (Abcam), mouse monoclonal anti-NeuN (Millipore), mouse monoclonal anti-TuJ1 (Millipore), rabbit polyclonal anti-phospho-Smad2/3 (Cell Signaling), rabbit polyclonal anti-Aquaporin 4 and Fluorescein Lycopersicon Esculentum Lectin-FITC (Vector Laboratories). Guinea pig anti-NG2 was

a kind gift from Dr. W. Stallcup. Alexa Fluor® (Life Technologies) secondary antibodies were used for detection of the primary antibody.

For endothelial proliferation assays, the Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Life Technologies) was used according to the manufacturer's instructions.

Neural stem cell cultures, proliferation and differentiation

Neural stem cell cultures (neurospheres) were performed as described previously (*20*). For proliferation, neurospheres were maintained in serum-free medium containing EGF and bFGF (both 20ng/ml). For the differentiation assay, neurospheres were trypsinized and plated on poly-lysine/laminin coated coverslips in serum-free, growth-factor-free medium as described previously (*20*).

Endothelial cell cultures

Primary mouse brain microvascular endothelial cells were purchased from Cell Biologics and maintained in Endothelial Cell Medium according to vendor's instructions. For proliferation assays, cells were trypsinized and seeded in a gelatin-coated 96-well plate at a density of 2000 cells/well. Complete medium was replaced with the experimental growth medium 12 hours after plating. EdU was added at a concentration of 10μM four hours prior to fixation with 4% paraformaldehyde. Cells were stained with the EdU kit and the nuclear dye Hoechst 33342.

For pSMAD assays, primary brain vessel endothelial cells were treated as indicated, in the presence of sodium orthovanadate, to preserve phosphorylation for 30 minutes, fixed with 4% paraformaldehyde, subjected to immunocytochemical analysis, and imaged using the Operetta high content imaging microscope (Perkin Elmer). Automated image analysis was performed using Columbus software (Perkin Elmer).

Olfactory sensitivity assay

This test is designed to detect differences in olfactory sensitivity between two groups of mice. The olfactory sensitivity assay was performed as previously described (*18*). Before the assay, control mice were exposed to different sets of odorants in order to measure the spontaneous response to the different odorants and concentrations. For the assay, every naïve parabiont (3 days after pair separation) was placed in a new, clean cage and left for 15 min for habituation. Then, different dilutions of the odorant (Peppermint, Sigma-Aldrich) ranging from 10 to 10,000 were infused on a disk and the disk placed in the cage across form the mouse. To avoid bias and to ensure that these results were not due to habituation to the odorant, the experiment was conducted both from high to low dilution and the reverse. The total time of exploration was measured, and data were analyzed by Graph Pad Prism.

Perfusion MRI

Relative cerebral blood flow (CBF) was measured by using a flow-sensitive alternating inversion recovery (FAIR) sequence (*34*) on a Bruker Pharmascan 4.7T MRI using endogenous water protons as a tracer (TR = 16000 ms, TE = 23 ms, 64 x 64 x 1 matrix size, 0.625 mm x 0.625 mm x 2 mm voxel size) and with a Bruker 3.7 cm diameter transmit-receive coil to fit both mice in the scanner. This arterial spin labeling technique performs two echo planar images (EPI) with both flow sensitive and flow

insensitive EPI scans. During the inversion delay time after slice-selective inversion, fully magnetized blood protons move into the imaging slice and exchange with tissue water protons. The difference between these two images is directly proportional to relative blood flow. In addition, a T2-weighted (T2w) scan (TR = 2000 ms, TE = 20 ms, effective TE = 60 ms, 8 averages, 192 x 192 x 22 matrix size, 0.133 mm x 0.130 mm x 0.7 mm voxel size) was performed to select a slice in the subventricular zone. The geometry was customized for each of the parabiosed mice to ensure axial slice selection for the images. The FAIR CBF map for each mouse was fused to the T2w anatomical images in OsiriX (http://www.osirix-viewer.com). Using the T2w images as anatomical reference, fixed-size oval regions-of-interest (ROI) were drawn in the SVZs on the FAIR CBF maps to measure the mean image intensities in the SVZs. The mean image intensities of the ventricles were used as the background. 3D images were generated using Amira (VSG, Burlington, MA) after segmentation for the brain on T2w images and fusing with the FAIR CBF maps.

Volumetric 3D assays

To measure the volume of blood vessels, 100µm-thick sections were imaged using a Zeiss LSM 510 inverted confocal microscope in order to create z-stacks. The z-stacks were analyzed using Volocity 3D Image analysis software (Perkin Elmer) to create three-dimensional angiograms and calculate the total volume.

Imaging

Imaging was performed using a Zeiss LSM 510 inverted confocal microscope, a Nikon Eclipse Ti microscope and the Operetta High Content Imaging System (Perkin Elmer).

Quantification of immunostaining

In all experiments, quantification was performed blinded: each experiment was assessed a code which was revealed after analysis. For the *in vivo* experiments, **n** represents the number of mice of each experimental group. For each mouse, 8-10 different sections comprising the same area of the SVZ (as shown on Fig. S2) were analyzed. Quantification of all figures was performed per field of view except for Olig2, which was measured only in the SVZ as marked by $GFAP^+$ cells. Sox2 quantification in Fig.4C was performed using a rectangular shape that was copied in all the images. Only cells inside the shape were counted.

In endothelial cell proliferation assays, images of the whole well (12-20 fields/well, 20x magnification) were acquired using the Operetta high content automated imaging microscope and were analyzed using Columbus Image Analysis Software (Perkin Elmer). Each *in vitro* experiment was repeated at least 3 times, except for the experiment in Fig. S12, which was performed once.

Statistical analysis

All statistical analyses were performed using Graph Pad Prism software, with Student's t-test assuming two-tailed distribution and equal or unequal variances or ANOVA depending on the experiment. Statistical significance was assigned for $p<0.05$; results are shown as standard error of the mean.

Supplementary Figures

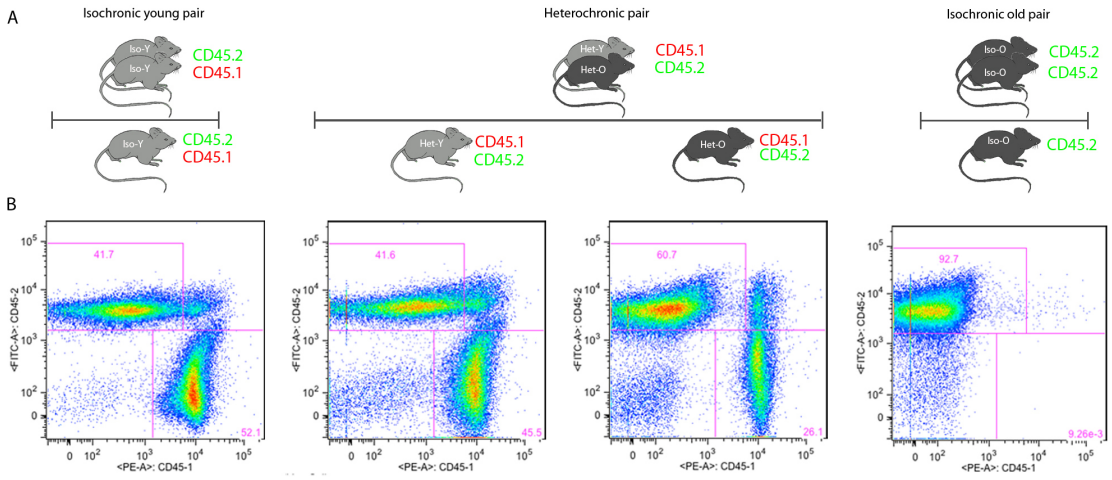

Fig. S1.

(**A**) Graphical scheme representing parabiotic pairs of young (2 months) and old (15-16 months) mice that were joined for 5 weeks. To confirm parabiotic cross-circulation by blood chimerism, we used young mice carrying the congenic marker CD45.1 and old mice carrying the congenic marker CD45.2 in the heterochronic pair and young mice carrying either CD45.1 or CD45.2 in the Iso-Y pair. (**B**) Flow-cytometry analysis of the chimeric blood from Iso-Y, Het-Y, Het-O and Iso-O mice.

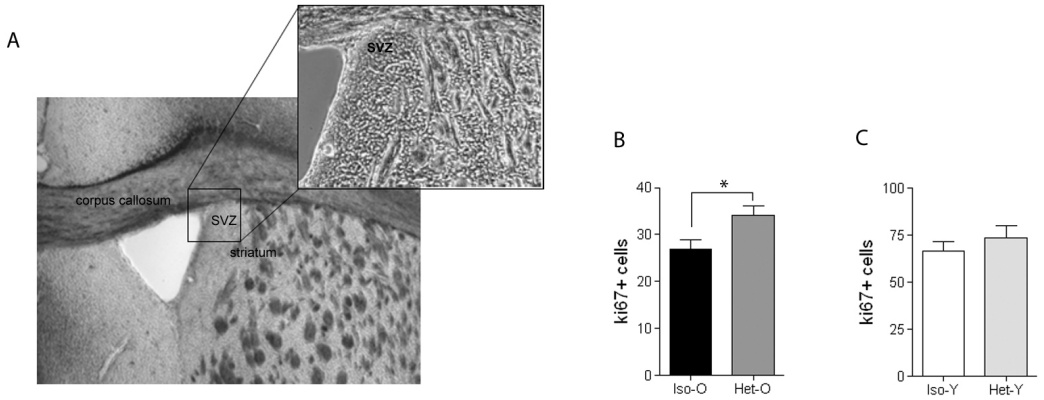

Fig. S2.

(A) Bright field images of a coronal section depicting the SVZ area (insert). **(B, C)** Quantification of Ki67^{+} proliferative cell populations in the SVZ area in old (B) and

young (C) parabionts (n=9 animals per condition, *p<0.05). Data shown as mean±S.E.M; statistical analysis by Student's t-test.

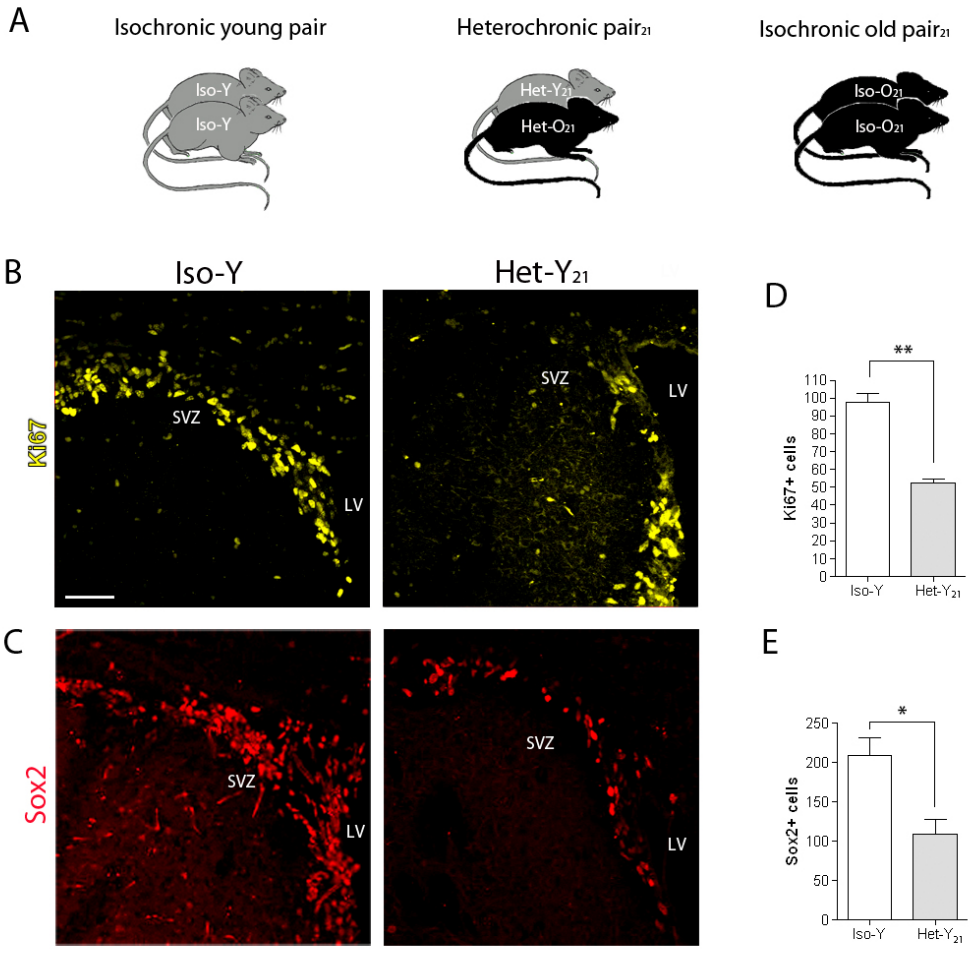

Fig. S3.

(**A**) Graphical scheme representing parabiotic pairs of young (2 months, CD45.1) and old (21 months, CD45.2) mice joined for 5 weeks. (**B, C**) Confocal images of coronal sections of the SVZ labeled for Ki67 (B) and Sox2 (C). Scale bar: 50µm. (**D, E**) Quantification of Ki67+ (D) and Sox2+ (E) cells in the SVZ area of young parabionts (n=4 animals per condition , *p<0.05, **p<0.01). Data shown as mean±S.E.M; statistical analysis by Student's t-test.

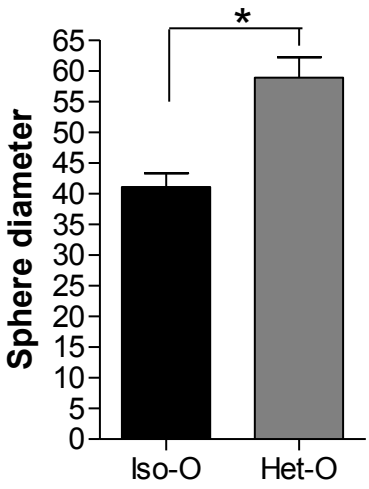

Fig. S4.

Histogram showing the difference in size of neurospheres derived from either Iso-O or Het-O mice. Neurospheres were passaged at least once (n=15, **p≤0.01). Data shown as mean±S.E.M; statistical analysis by t-test.

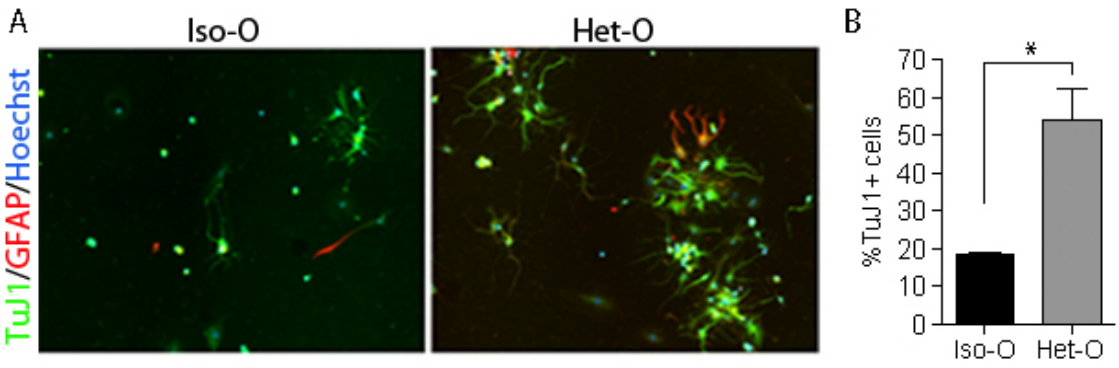

Fig. S5. Heterochronic parabiosis induces neuronal differentiation in vitro. (**A**) Representative image of a neurosphere differentiation assay using neurospheres derived from either Iso-O or Het-O mice. **(B)** Histograms showing the difference in production of TuJ1+ neurons (n=12, *p≤0.05). Data shown as mean±S.E.M; statistical analysis by t-test.

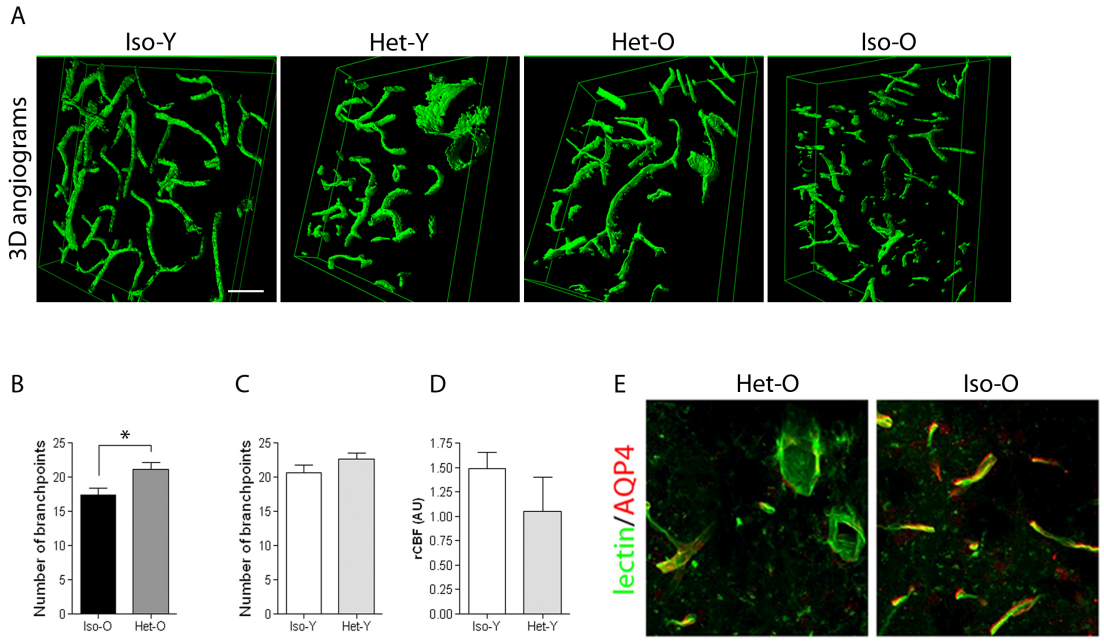

Fig. S6.

(**A**) 3-D reconstructions (angiograms) of the SVZ vasculature generated by confocal imaging of 100µm thick sections and processed with Volocity software. (**B**, **C**) Quantification of blood vessel branching per field in old (B) and young (C) parabionts (n_{old}= 8, n_{young}=5 animals per condition, *p<0.05). (**D**) Perfusion MRI measurements of cerebral blood flow in the SVZ region of young (Iso-Y versus Het-Y) parabionts (n=4 animals per condition). Data shown as mean±S.E.M; statistical analysis by t-test. (**E**) Confocal images of coronal SVZ sections showing double-labeling with lectin and aquaporin-4. Scale bar: 50 µm.

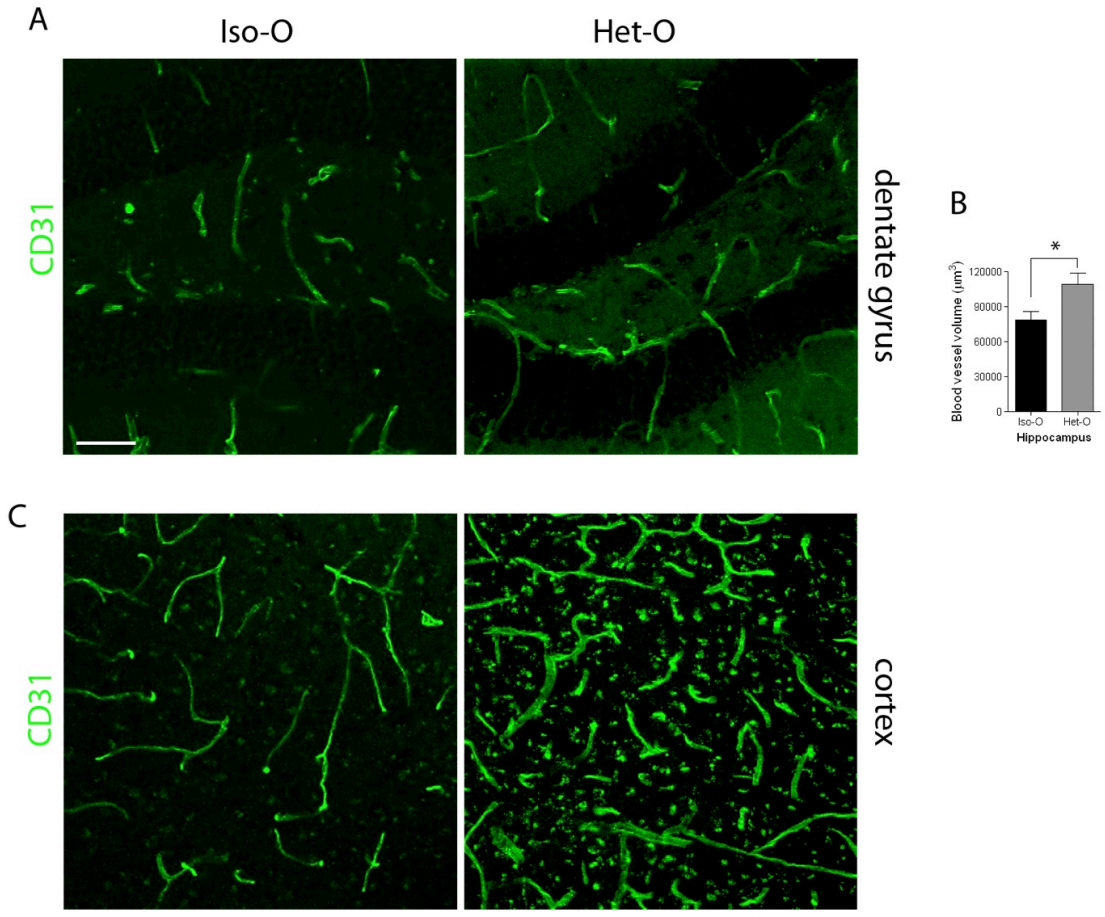

Fig. S7.
(A, C) Confocal images of coronal sections of hippocampal dentate gyrus (A) and cortex (C) labeled with a CD31 antibody. Scale bar: 50 µm. (**B**) Volumetric analysis of 3D angiograms generated from z-stacks of 100µm thick sections of the dentate gyrus of old isochronic and heterochronic parabionts (n= 7 animals for each experimental group, *$p<0.05$). Data shown as mean±S.E.M; statistical analysis by Student's t-test.

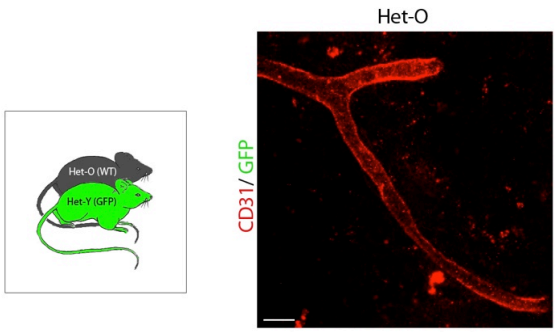

Fig. S8.
Heterochronic parabiosis between 2-month old GFP mice and 15-month old non-GFP mice. High magnification confocal image of Het-O blood vessels showing that there is no detectable contribution of circulating GFP^+ cells to brain vasculature. Scale bar 20µm.

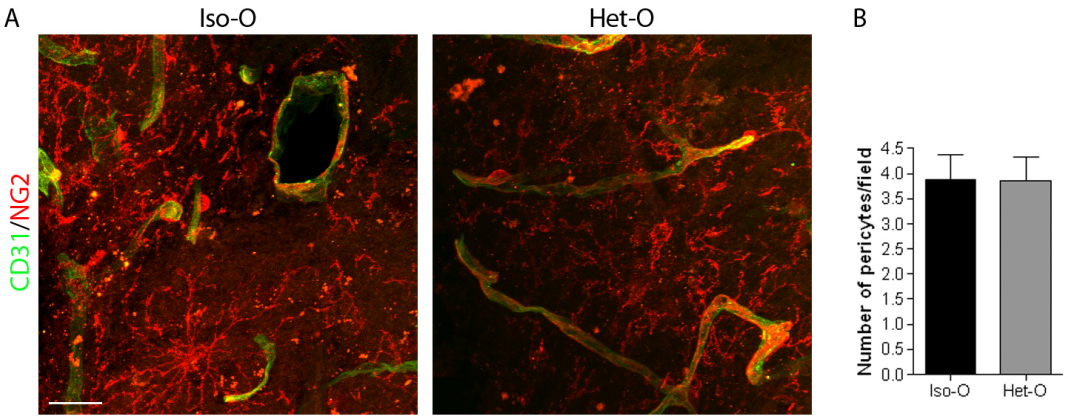

Fig. S9.
(A) Confocal images of SVZ coronal sections double-labeled with NG2 and CD31 to mark pericytes. Scale bar: 20 µm. **(B)** Quantification of $NG2^+/CD31^+$ cells in the SVZ area of old parabionts (n=4 animals per experimental group). Data shown as mean±S.E.M; statistical analysis by Student's t-test.

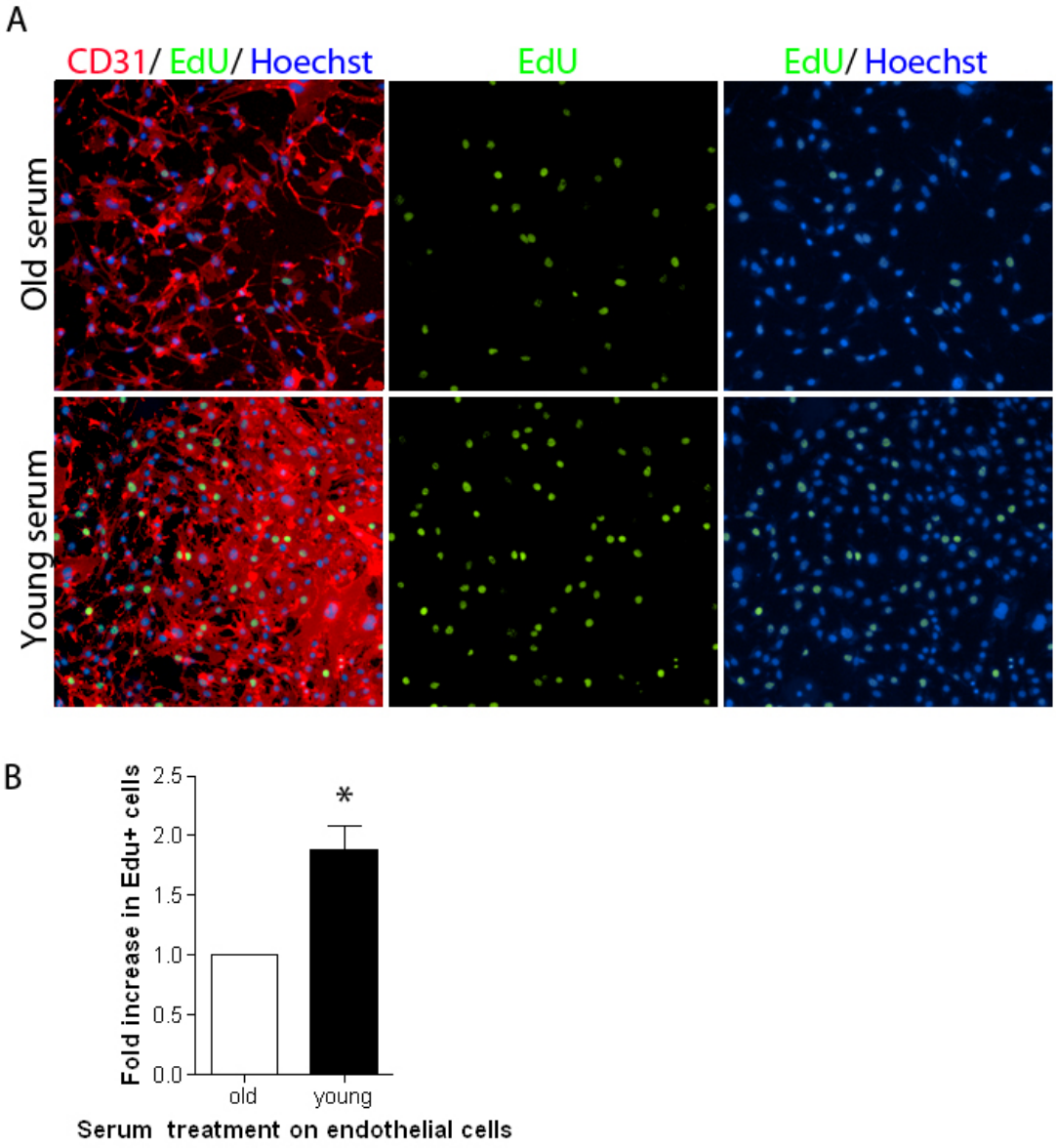

Fig. S10.
(A) Representative images of primary brain capillary endothelial cell cultures treated with serum-free media supplemented with either young or old mouse serum for 2 days and then pulsed with EdU for the last 4h of the treatment. (**B**) Quantification of the images (n=3 independent experiments, 4 wells per experiment and 12 fields of view per well, *p≤0.05). Data shown as mean±S.E.M; statistical analysis by pair-wise t-test. Scale bar: 50 μm.

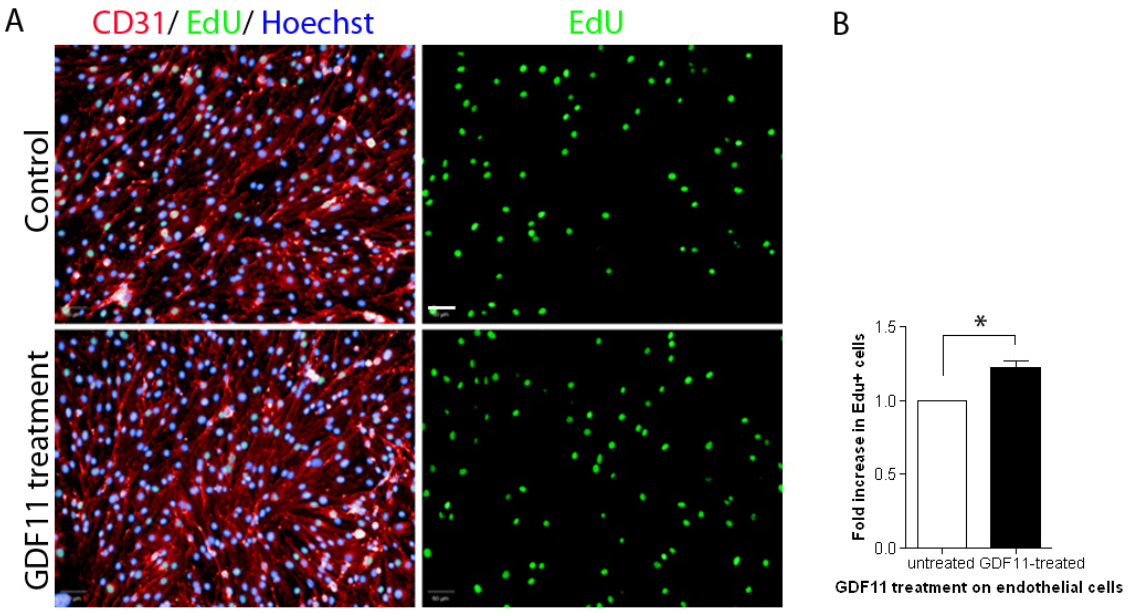

Fig. S11.
(A) Images of primary brain endothelial cell culture after treatment with GDF11 for 6 days and EdU for the last 4 hours. Cells were labeled with CD31, EdU and Hoechst. (**B**) Quantification of the images (n=3 independent experiments, 4 wells per experiment and 12 fields of view per well *p<0.05). Data shown as mean±S.E.M; statistical analysis by pair-wise t-test. Scale bars: 50µm.

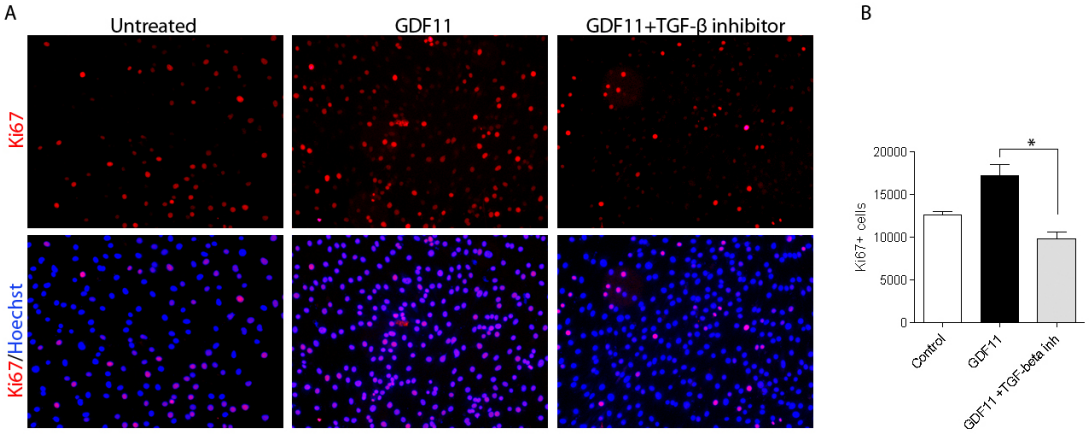

Fig. S12.

(A) Images of primary brain endothelial cell culture after treatment with GDF11 (40ng/ml) or GDF11 (40ng/ml) together with TGF-β inhibitor (SB431542, 10μM) for 5 days or untreated. Cells were labeled with Ki67 and Hoechst. (**B**) Quantification of the images (n=1 experiment, 3 wells per experiment and 20 fields of view per well *$p<0.05$). Data shown as mean±S.E.M; statistical analysis by pair-wise t-test. Scale bar: 50μm.